Maple Sirup. XXI. The Effect of Temperature and Formaldehyde on the Growth of Pseudomonas geniculata in Maple Sap

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SUMMARY

The growth of *Pseudomonas geniculata* in sterile maple sap in the presence and absence of 5 ppm formaldehyde at several temperatures was investigated. At 27°C, the growth of inocula as low as 10^3 cells/ml was rapid, and the effect of formaldehyde, if any, was negligible. At 7°C, the temperature slowed the growth of the bacteria, particularly in cultures inoculated with 10^3 or 10^4 cells/ml. Formaldehyde further inhibited the cultures, initially causing a decrease in the number of viable organisms and lengthening the lag period before growth began again. This was particularly noticeable in the cultures with the smaller inocula.

Tapholes for the collection of sap from maple trees are liable to infection by adventitious organisms. Naghski and Willits (1955) demonstrated that "prematurely dried-up" tapholes, a condition in which sap flow diminishes or ceases completely, are heavily contaminated with bacteria. Sheneman *et al.* (1959) found a high degree of correlation between the occurrence of large populations of bacteria early in the sap season and low yields of sap. Since the premature stoppage of sap flow results in serious economic losses of the maple sirup crop, an intensive investigation was carried out to find inhibitors for bacterial growth in sap. Paraformaldehyde (a polymer of formaldehyde) has proven the most promising compound in controlling the growth of microorganisms in a taphole (Sheneman *et al.*, 1959; Costilow *et al.*, 1962).

Formaldehyde, the active ingredient of paraformaldehyde, is a well known bacterial inhibitor. There is, however, a great deal of variation in the reports of the action of formaldehyde on microorganisms, due, perhaps, to the differences in the experimental conditions and organisms used. McCulloch and Fuller (1941) found that 1% formalin inhibited the growth of *Erysipelothrix rhusiopathiae* for only 24 hours after 10 minutes of exposure, and it required 30 minutes of exposure for a 4% solution of formalin to kill the organisms. Other studies (McCulloch, 1945) also used concentrations of formaldehyde as great as 5% to prevent the growth of a variety of organisms. Temperature, too, affects the activity of formaldehyde; McCulloch and Costigan (1936) reported that a solution of formaldehyde having germicidal activity at 40°C may be of questionable value at 20°C and almost inert below 10°C.

Pseudomonas geniculata is the organism most frequently isolated from maple sap. Costilow *et al.* (1962), in a report issued while this paper was in preparation, indicated that the growth of this organism, at an inoculum level of 10^4 organisms/ml, is completely inhibited by 30 ppm formaldehyde, and some inhibition occurs at lower concentrations.

The quantity of dissolved formaldehyde in sap collected from tapholes containing paraformaldehyde pellets is 5 ppm or less in approximately 92% of the saps studied (actually about 70% of the saps contained 1 ppm or less of formeldehyde) (Costilow

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et al., 1962). The temperature conditions under which maple sap is collected are usually below 10°C. Thus, according to the literature, the conditions in which paraformaldehyde is used in maple tapholes should not be conducive to inhibition of bacterial growth in the maple sap after it leaves the taphole.

This report describes a limited study of the effect of the specific concentration of formaldehyde most likely to be encountered in the field, namely 5 ppm, on the growth of *Pseudomonas geniculata* at 7 and 27°C. It was important that this concentration of formaldehyde be studied since it is anticipated that maple sap will eventually be used in controlled fermentations to yield sirups of desired properties.

MATERIALS AND METHODS

Ps. geniculata, strain 4, was isolated from maple sap (Naghski and Willits, 1955) and has been shown to produce maple flavor precursors in sterile sap through controlled fermentation. The organism was cultured in a medium containing maple sap sterilized by autoclaving 15 min at 121°C and decanted under aseptic conditions from the heat-precipitable material. To this sap was added a quantity of sterile Difco (no endorsement implied) yeast extract solution to give a final concentration of 0.5% in the medium. After incubation at room temperature (25°C) for approximately 48 hr the cells were collected by centrifugation and suspended in sterile distilled water. Appropriate dilutions were made in sterile distilled water.

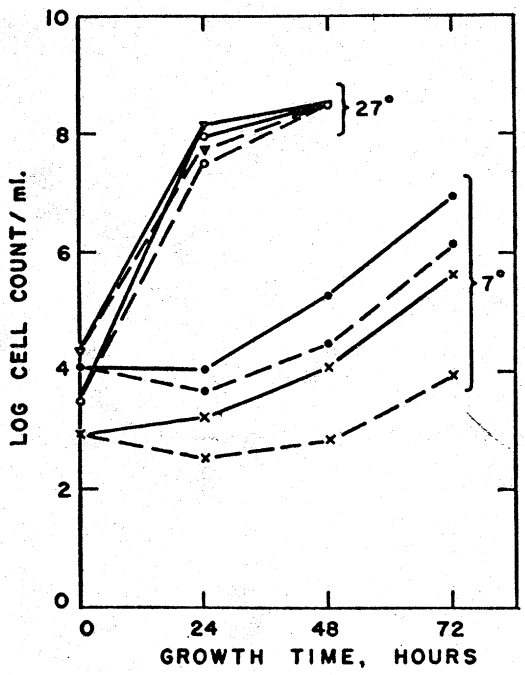

Fig. 1. The effect of temperature on formaldehyde inhibition of the growth of *Ps. geniculata.* Control (no formaldehyde), ———; 5 ppm formaldehyde, — — — —.

Erlenmeyer flasks, of 500 ml capacity, were charged with 200 ml of sterile maple sap and inoculated with cell suspensions to give the required cell concentration. One hundred ml of the suspensions were transferred to other 500-ml Erlenmeyer flasks and received sufficient formaldehyde to give a final concentration of 5 ppm. The pairs of inoculated flasks (with and without formaldehyde at each inoculum level) were incubated at 7°C or at 27°C without agitation or aeration.

Flasks were sampled immediately after inoculation and every 24 hr for the appropriate time period. The samples, after dilution in sterile distilled water, were plated on tryptose-glucose-beef extract agar (Difco) and incubated at 27°C. Counts were made after 48 hr of growth.

RESULTS

Ps. geniculata was inoculated into sets of t[illegible] medium to give population levels of 10^3 and 1[illegible] organisms/ml. These flasks were incubated at 27 or 7°C. Fig. 1 shows the results of the effect of formaldehyde and temperature on the growth of the bacteria. At 7°C, growth in the control flasks (without formaldehyde) was slow, showing an increase only by the second 24-hr period. Maximum growth was not attained even after 72 hr of incubation. In the presence of formaldehyde there was an initial decrease in the number of viable cells. The culture containing, originally, 10^4 cells/ml appeared to recover after 24 hr and the growth rate then paralleled that of the control culture. In the 10^3 cells/ml culture, however, the inhibitory action of the formaldehyde appeared to be greater, and the cell count did not increase appreciably until after 48 hr of incubation.

By contrast with the growth pattern at 7°C, growth at 27°C was almost explosive. Maximum growth was attained in 24 hr or shortly thereafter in both the control and formaldehyde-treated cultures. There is, however, some indication that the formaldehyde may have initially inhibited growth of the cells, since the treated-cell counts at 24 hr are somewhat less than the control counts. Although no viable counts were made before the 24-hr sampling, so the pattern of early growth is not known, it may be that even at this temperature, which favored the rapid growth of the or-

ganisms, the formaldehyde initially affected the cells in a manner similar to its action at 7°C.

Since formaldehyde inhibited the growth of *Pseudomonas* at 7° when a small inoculum was used, the effect on larger inocula was also investigated. Maple sap media, with and without formaldehyde, were inoculated to contain 10^3, 10^4, 10^5, and 10^6 organisms per ml. Growth of these cultures at 7° is shown in Fig. 2. The control cultures originally containing 10^5 and 10^6 organisms/ml grew within 24 hr, indicating that the low temperature had less effect on the continued growth of large numbers of organisms than on cultures containing fewer than 10^4 cells/ml. The presence of 5 ppm formaldehyde, however, was still effective against the higher-inocula cultures, inhibiting growth for at least 24 hr.

DISCUSSION

The bacteriostatic, or bactericidal, activity of a compound must be considered in relation to many factors: the organisms involved, the numbers of organism used, concentration of the compound, composition of the medium, and the temperature of incubation. The effect of formaldehyde, an accepted bacterial inhibitor, has been variously assessed, primarily because of variations in the testing conditions. Since this inhibitor has been shown to reduce the degree and rate of contamination of tapholes in maple trees, the effectiveness of low concentrations of formaldehyde on the growth of bacteria in collected sap was evaluated under conditions similar to those likely to prevail in the field.

Ps. geniculata, isolated from sap, was selected as the test organism. Although some yeasts, molds and other bacteria have been found in maple sap this species is the predominating organism. Formaldehyde, at a concentration of 30 ppm, has been shown to be bactericidal to *Ps. geniculata,* but only under laboratory conditions (Costilow *et al.,* 1962). Under field conditions the organisms would be growing in maple sap, which is a minimal medium for maintaining the growth of this organism, and at temperatures of approximately 0°C. In a study of the solution rate of paraformaldehyde pellets designed to be used in the tapholes, the formaldehyde concentration in stationary water approached the 30-ppm lethal dose in 24–48 hr, whereas, in a simulated taphole, flowing water contained about 5 ppm of the

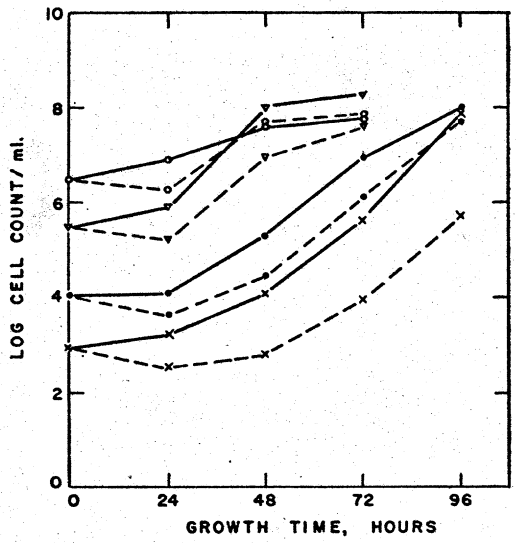

Fig. 2. The effect of inoculum size on the formaldehyde inhibition of the growth of *Ps. geniculata* at 7°C. Control (no formaldehyde), ———; 5 ppm formaldehyde, – – – –.

inhibitor (Costilow *et al.,* 1962). Under actual conditions, collected sap was found to contain less than 5 ppm formaldehyde in 92% of the samples tested. Thus, from the literature reports, it would appear doubtful that the formaldehyde concentration in the field would be great enough to inhibit the bacteria, although actual trials did show substantial inhibition of taphole contamination. The findings of this limited study indicate that two factors are involved in inhibition of the growth of small populations of bacteria. In the control cultures, low temperatures result in a decrease in the over-all growth rate and there is a definite lag period of about 24 hr before growth occurs.

The inhibitory effect of the formaldehyde can be distinguished from temperature-induced inhibition. At the low inocula levels, particularly at 10^3 cells/ml, the number of viable cells decreases in the first 24 hr, and the amount of growth is appreciably less than in the control cells exposed to low temperature only.

(Low viable counts could possibly be due to a carry-over of formaldehyde to the plating medium. However, cultures were diluted at least 1:100 before plating, thus reducing the maximum amount of formaldehyde possibly carried over to 0.05 ppm. Since 0.5-ml samples of the dilutions were

plated in approximately 10 ml agar medium, the final formaldehyde concentration per plate was less than 0.025 μg.) Even at the higher inocula levels, where the effect of low temperature is not so evident, the cells exposed to formaldehyde undergo an initial period during which no increase in the growth rate occurs. Although the growth rate of the bacteria is very rapid at 27°C, there appears to be some indication that the formaldehyde may have had a transient inhibitory effect readily overcome by the bacteria.

It is not possible to decide from the data whether the action of low temperature and formaldehyde in inhibiting the bacterial growth was additive or synergistic. However, it is suggested that one effect of the lower temperatures in reducing the metabolic activity of the cells is to render them more susceptible to the action of the formaldehyde. This appears to be particularly true at the lower inocula levels.

It is interesting to note that the inhibitory activity of formaldehyde at 5°C is contrary to the findings of McCulloch and Costigan (1936) that lower temperatures decreased the activity of this compound.

Another consideration of this study was the possible effect of the formaldehyde inhibition of bacterial growth on the flavor of maple sirup. Sirup made from sterile sap lacks the full characteristic maple flavor, and some bacterial action (by the proper bacteria) is necessary for complete development of this unknown factor. Reduction of the bacterial population with formaldehyde could result in the production of flavorless sirup. The data indicate, however, that formaldehyde, under the conditions stated, is bacteriostatic, inhibiting growth temporarily. Full development of the flavor factors can be expected to occur in the treated sap, either by outgrowth of the normal flora of the sap or by controlled fermentation with selected bacterial strains.

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